Unraveling clonal heterogeneity in acute myeloid leukemia

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Summary, discussion, future perspectives, and concluding remarks

Summary

Acute myeloid leukemia (AML) is a hierarchical disease with leukemic stem cells (LSCs) that give rise to immature leukemic blasts that lack the capability to differentiate in mature blood cells. In addition, multiple genetically distinct subclones can co-exist within an AML patient. In this thesis, we made use of the leukemic plasma membrane (PM) surfaceome to study this clonal heterogeneity, we studied the functional role of leukemia-enriched PM protein interleukin-1 receptor accessory protein (IL1RAP) and further improved our previously established humanized scaffold niche xenograft mouse model by introducing interleukin-1 (IL1) and thrombopoietin (TPO).

Our previously established humanized scaffold niche xenograft mouse model allows us to study primary AML samples *in vivo* in a human microenvironment. In **chapter 2**, we genetically engineered mesenchymal stromal cells (MSCs) to overexpress IL3 and TPO and used these MSCs to build a humanized BM scaffold. The IL3- and TPO-producing MSCs maintained their ability to differentiate into bone, adipocytes, and other stromal components. Primary AML cells of three individual patients could readily engraft in these cytokine-expressing niches, including a biphenotypic acute leukemia (BAL) patient, from which the myeloid compartment model was better preserved compared to the previous humanized scaffold niche model. Transplantation of MLL-AF9 transduced cord blood (CB) $CD34^+$ in mice with human cytokine-expressing niches resulted in reduced scaffold engraftment but significantly increased myeloid output of cells that engrafted in the mouse compartments including BM, spleen and liver. The high local levels of IL3 within the scaffold might result in stem cell differentiation and thereby reduced engraftment. Titrating the levels of IL3 and TPO might be needed to further improve this cytokine-expressing BM scaffold niche. This study showed that with relatively simple genetic editing of human MSCs, important niche factors can be studied in context of a human microenvironment.

Chapter 3 studies the intra-tumor heterogeneity of AML caused by clonal evolution. We first identified 50 plasma membrane (PM) proteins that were specifically (over) expressed on primary AML $CD34^+$ cells compared with normal $CD34^+$ cells. These proteins were correlated with different leukemia subtypes and leukemia-associated mutations. We included 7 leukemia-enriched PM proteins in the routinely diagnostics and showed that these proteins could define a leukemia-associated phenotype in the majority of patients. A combinatorial approach comparing the expression of multiple leukemia-enriched PM proteins allowed the identification and isolation of genetically distinct AML subclones. Next, we studied these AML subclones and showed that they differ in their chromatin organization, transcription factor networks, gene expression, engraftment behavior, and drug sensitivity. Finally, we showed that these leukemia-enriched markers can be used to track leukemic clones longitudinally. The results in this chapter provides tools to separate

and study individual AML subclones.

In **chapter 4** we focused on the PM protein IL1RAP. IL1RAP was found specifically expressed on the outer membrane of AML $CD34^+$ cells and not on normal $CD34^+$ cells. The intracellular role of IL1RAP in AML is mostly unknown, therefore, we studied the downstream signaling of IL1RAP in primary AML cells. Activation of IL1RAP positive leukemia cells with IL1 resulted in the induction of multiple chemokines and an inflammatory secretome via the p38 MAPK and NFκB signaling pathways. In contrast to previous studies, we did not observe any negative growth phenotype after knockdown of IL1RAP. Intriguingly, the inflammatory secretome did not affect AML cell survival in an MSC co-culture settings, whereas the secretome did affect the growth of normal $CD34^+$ cells. This phenotype was only observed in context of an MSC stromal layer and suggests that direct crosstalk between AML cells and MSCs is essential for the observed effect. Interfering with the IL1-IL1RAP signaling might facilitate healthy hematopoietic recovery and should therefore be therapeutically exploited.

Finally, **chapter 5** discusses the results described in chapter 2,3, and 4 in context of the current literature. Possible future directions are discussed supported by preliminary data. The chapter closes with the concluding remarks of this thesis.

Discussion and future perspectives

Identification of leukemia-enriched PM proteins

PM proteins are responsible for the communication with neighboring cells in the bone marrow (BM) niche via direct and indirect signals. The identification of the PM surfaceome of AML cells greatly improves our knowledge of these BM niche interactions. Multiple transcriptome datasets are available of studies that compared primary AML cells with healthy controls [1-4], whereas the amount of studies that identified leukemia PM markers at the protein level are limited [5-8]. We performed full label-free quantitative proteome analysis on immature AML blasts and healthy peripheral blood stem cells (PBSCs). Principle component analysis (PCA) of all proteins annotated in at least 90% of the samples (6025 proteins) showed separated clustering of PBSC $CD34^+$ cells compared to AML $CD34^+$ cells (Figure 1A, left panel). Similar results were obtained for proteins specific to the PM (849 proteins) (Figure 1A, right panel)[9]. AML $CD34^+$ cells were enriched for gene ontology (GO) terms involved in cell cycle, metabolic processes and cytoskeleton organization compared to healthy PBSC $CD34^+$ cells (Figure 1B). Subsequent analysis of the PM proteome identified 50 PM proteins that were specifically enriched in AML [8]. The majority of proteins we identified were overexpressed in a subset but not all AML patients and many, but not all, were also found to be upregulated at the transcriptome level. Previously, Bonardi *et al*. identified 59 leukemia-enriched PM protein candidates determined by surface-specific proteomics of 2 primary AMLs combined with transcriptome analysis on a large cohort of normal bone marrow (NBM) and AML samples published by de Jonge *et al* [3, 5]. We found substantial overlap (12 proteins) of these previous identified leukemia-enriched PM proteins with the 50 proteins identified in this thesis. Candidate markers identified by the proteomics in this thesis has been performed in a much larger cohort with multiple different AML subtypes and thereby might also include subtype-specific PM proteins. A recent study by Perna *et al.* also defined a set of leukemia-enriched PM proteins [7]. They performed surface-specific proteomics on 6 AML cell lines and supplemented this list with previously performed proteomics on cell lines and 70 candidates proposed by others. They compared these proteins with multiple transcriptome datasets of healthy immature hematopoietic cells and non-hematopoietic tissues in order to prevent off-target toxicity of antibody-based therapies. In total they propose 30 potential candidates overexpressed in AML and low or not expressed in non-hematopoietic tissue. Some proposed candidates overlap with the PM proteins identified in this thesis although the majority does not, likely as of differences in cell source (cell lines versus and primary AML) and technical approach (PM proteomics versus total proteomics).

We also performed proteomics on 6 cell lines (HL60, MOLM13, NB4, TF1, THP1 and K562) on the same platform as we used for the primary material. Surprisingly, PCA on all

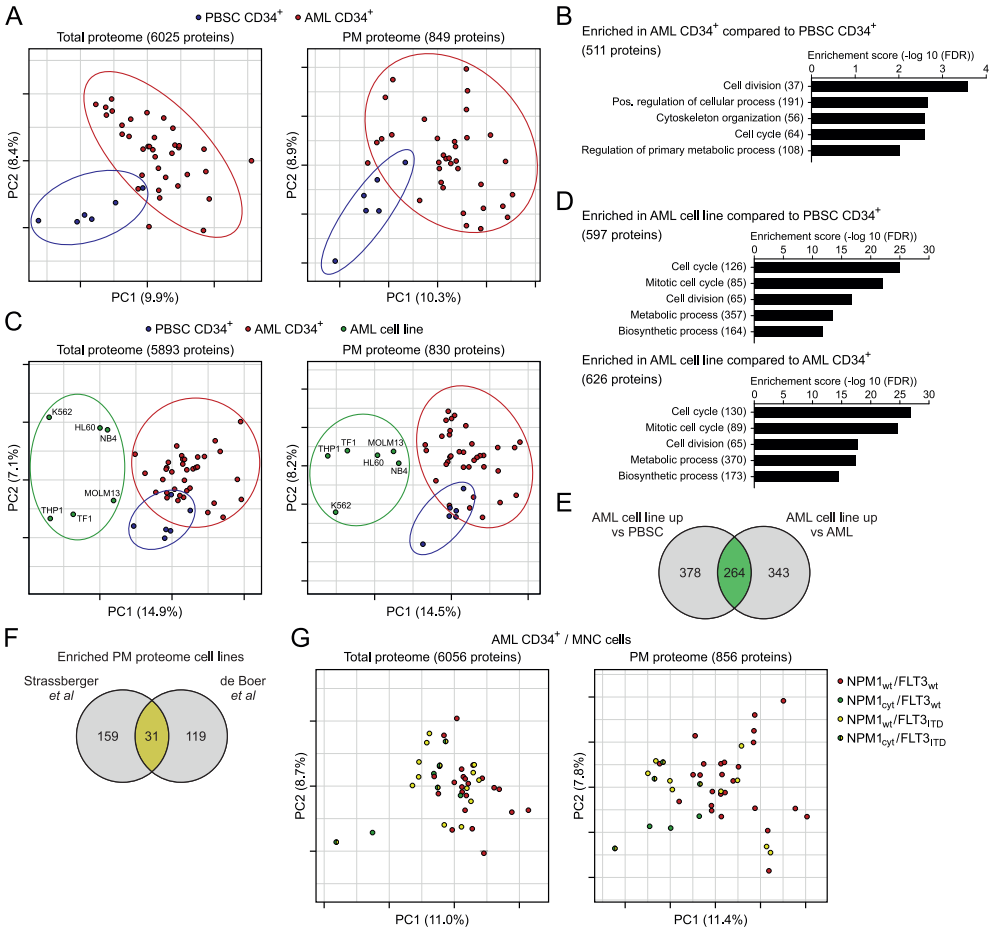

Figure 1. Full quantitative proteomics analysis of AML

(A) PCA analysis of total proteome (left) and PM-specific proteome (right) in PBSC CD34⁺ cells (blue, n=6) and AML CD34⁺ cells (red, n=36). (B) GO analysis of proteins >2 fold upregulated (p<0.05) in AML CD34⁺ cells compared to PBSC CD34⁺ cells. (C) PCA analysis of total proteome (left) and PM-specific proteome (right) in PBSC CD34⁺ cells (blue, n=6), AML CD34⁺ cells (red, n=36) and AML cell lines (green, n=6). (D) GO analysis of proteins >2 fold upregulated (p<0.05) in AML cell lines compared to AML CD34⁺ cells (top panel) and PBSC CD34⁺ cells (bottom panel). (E) Overlap of proteins upregulated in cell lines compared to AML CD34⁺ cells or PBSC CD34⁺ cells as described under D. (F) Overlap of AML cell line enriched PM proteins identified by Strassberger *et al.* and de Boer *et al.* [6, 8]. AML cell line PM proteins were compared to PMN and PBSC CD34⁺ cells, respectively. (G) PCA analysis of total proteome (left) and PM-specific proteome (right) in AML CD34⁺/MNC cells (n=42). Proteins were associated to the PM as determined previously by Uhlen *et al.* for all panels [9]. FDR, False discovery rate.

annotated proteins in at least 90% of the samples (5893 proteins) revealed that healthy CD34⁺ and AML CD34⁺ cells are more alike compared to AML cell lines (Figure 1C, left panel) [9]. Proteins that were significantly overexpressed in AML cell lines compared to PBSC CD34⁺ cells or AML CD34⁺ cells were highly enriched for processes associated with cell cycle and metabolism (Figure 1D). We observed substantial overlap between the proteins upregulated

in cell lines compared to PBSC CD34$^+$ cells and upregulated proteins compared to AML CD34$^+$ cells suggesting this is not disease-related but rather a difference between cell lines and primary material in general (Figure 1E). Intriguingly, also the PM proteome of cell lines is significantly different from primary material suggesting that this fast cycling phenotype also effects the surfaceome (Figure 1C, right panel). Also Strassberger *et al.* performed surface-specific proteome analysis on AML cell lines (HL60, NB4, PLB985, THP1), one blast crisis chronic myeloid leukemia (BC-CML) cell line (K562) and compared this to a normal human granulocytic cell line (PMN) [6]. We found considerable overlap between the upregulated PM proteins identified by Strassberger *et al*. and the upregulated PM proteins we identified despite the fact that different healthy controls were used. Clearly, we showed that cell lines differ substantially from primary material and data obtained from cell lines might need further validation in primary material. Cell lines are immortalized and might therefore be different in their proteome compared to the primary cells from which they were derived initially. Comparative studies of cell lines and primary cells showed that cell lines lose specific functions that were seen in primary cells and shift their resources into functions associated with proliferation [10]. In time, these cell lines might undergo a Darwinian selection of certain clones that do well in our culture systems.

Genetic aberrancies impact on chromatin accessibility and gene expression

Clustering analysis of AML patient samples only showed that the patients with a *nucleophosmin 1* (*NPM1*) mutation (*NPM1*$_{cyt}$) have a different proteome compared to *NPM1* wild-type (*NPM1-wt*) AML patients (Figure 1G). Important to note is that AMLs with a *NPM1*$_{cyt}$ mutation often have <1% CD34 positivity, therefore we performed proteomics on the mononuclear cell fraction (MNC) in 6 out of 7 *NPM1*$_{cyt}$ mutant cases whereas all other samples were enriched for CD34$^+$ cells. Some patients that had an internal tandem duplication (ITD) in *fms like tyrosine kinase 3* (*FLT3*) clustered together as well, although the differences with *FLT3* wild-type (*FLT3-wt*) patients were less clear compared to the proteome of *NPM1*$_{cyt}$ mutated AMLs (Figure 1G). These data suggest that genetic mutations can impose differences in total and PM-specific protein expression. Indeed, we observed multiple correlations of PM protein expression with specific genetic mutations. For example, CD25 and IL1RAP expression was increased in *FLT3-ITD* AMLs, CD97 was increased in *NPM1*$_{cyt}$ mutated AMLs and complex karyotype AMLs have reduced expression of T-cell immunoglobulin and mucin-domain containing-3 (TIM3), CD82 and CD123. It has been shown that FLT3-ITD result in constitutive activation of STAT5 and mitogen-activated protein kinase (MAPK) [11, 12]. This activation also results in the down-regulation of CCAAT/enhancer-binding protein alpha (CEBPA) and PU.1, two important transcription factors (TFs) that control monocytic and granulocytic differentiation, respectively [12]. In addition, the MAPK responsive transcription factor AP-1 has been identified as major driver of the FLT3-

ITD specific chromatin landscape [13].

Signal transduction networks initiated downstream of FLT3-ITD might instruct the observed differences in the transcriptome and proteome of *FLT3-ITD* AML patients compared to *FLT3-wt* AML patients. The identification and isolation of a *FLT3-ITD* and *FLT3-wt* subclone allowed us to study differences in the chromatin landscape and TF occupancy between genetically distinct subclones in an individual patient. RNA sequencing analysis showed that the *FLT3-ITD* subclone maintained an expression profile comparative with previous *FLT3-ITD* identified expression profiles [13, 14]. We observed limited but clear differences between chromatin accessibility of both subclones, which was associated with differences in TF occupancy throughout the genome. Similarly, we noticed subclone-specific chromatin accessibility, TF occupancy and gene expression in an AML patient that harbored a *NRAS* and *wilms tumor 1* (*WT1*) mutated subclone. *Ras* mutations have been associated with the proteasome machinery and interleukin-1 (IL1)/ nuclear factor kappa B (NFκB) signaling in lung cancers [15]. And *WT1* loss-of-function mutations and have been associated with site-specific in DNA hydroxymethylation, which resulted differential gene expression [16]. These data are in line with our transcriptome observations in the *NRAS* an *WT1* mutated subclones, which showed enrichment for IL1 mediated inflammatory processes and chromatin organization, respectively. Limited differences were observed in the chromatin accessibility in two other AML patients in which we could also separate distinct subclones. In one of these patients we separated a subclone with a mutation in *NRAS* at position 38 ($NRAS^{38G>A}$) from a subclone with a mutation in *NRAS* at position 183 ($NRAS^{183T>A}$). In the other patient we could separate a subclone with a duplication in *WT1* from a subclone with a deletion in *WT1*. These data suggest that different mutations in identical genes have limited impact on the chromatin landscape likely as they have similar biological consequences, at least in these 2 examples.

Clustering analysis of genome wide chromatin accessibility in the 8 different subclones isolated from 4 individual AML patients revealed that patient-specific chromatin accessibility is dominant over changes imposed by secondary driver mutations that defined the distinct subclones. The Bonifer lab previously generated chromatin accessibility maps of the blast population in a large cohort of AML patient samples [17]. We questioned whether the genetically distinct subclones we identified in 4 AML cases would cluster according to their common founder mutations or whether secondary subclone-specific mutations would be more dominant. In these 4 cases, it appeared that the common founder mutations in *CEBPA*, *runt-related transcription factor 1* (*RUNX1*) and *NPM1* dominated over the secondary mutations in *FLT3*, *NRAS* and *WT1* (Figure 2). Whether this is a common phenomenon needs to be addressed further in future studies in which more subclones will be analyzed in detail.

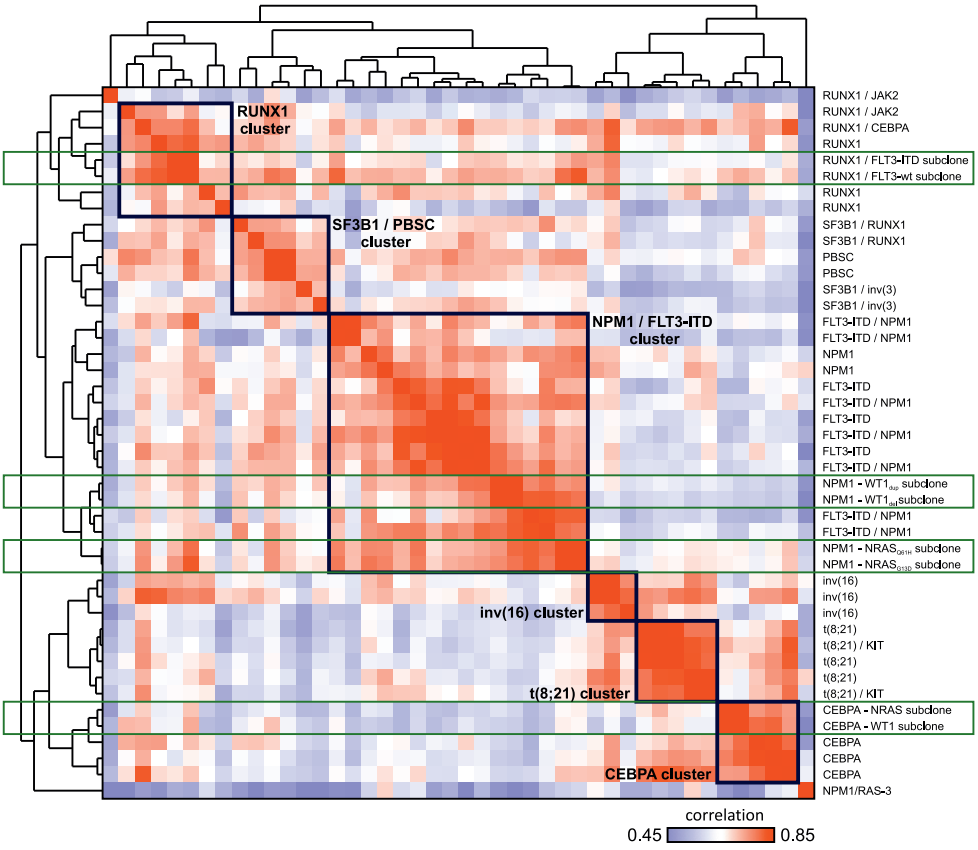

Figure 2. Chromatin accessibility in AML

Pearson correlation of chromatin accessibility measured by DNAse I Hypersensitive site mapping in AMLs and AML subclones carrying different mutations. Blue boxes indicate clusters of similarly mutated AML patients. Green boxes highlight isolated AML subclones of individual patients [8, 17]. The Bonifer lab is acknowledged for providing this specific figure.

Challenges in identification and isolation of genetically distinct AML subclones

Whole exome sequencing of *de novo* AMLs revealed that the majority of AML patients harbor multiple genetically distinct subclones [18-20]. We showed that genetic mutations resulted in altered protein composition on the PM, which allowed us to prospectively sort genetically and functionally distinct subclones [8]. Though, as seen with many newly developed tools, there are still challenges ahead that require further refinement and improvement.

AML patients can be relatively easily defined as monoclonal or polyclonal by mutation analysis of AML blasts. If mutations are present with variant allelic frequencies (VAFs) that are significantly different from 0.5 (1 allele mutated in all cells) or 1 (both alleles mutated in all cells), this must be as of the existence of multiple genetically distinct clones. Flow cytometry analysis of these polyclonal AMLs did not always result in differences in a selected

subset of leukemia-enriched PM protein expression. It might be that certain mutations have only limited or no impact on the PM protein expression. Alternatively, the selected subset of PM proteins might not allow separation of these subclones. For example, we made use of the TCGA dataset and tried to identify PM proteins specifically expressed in isocitrate dehydrogenase 1 (IDH1) and IDH2 mutated AMLs [18]. When we analyzed expression of 10 leukemia-enriched PM proteins we identified previously, we did not observe differences between IDH1/2 wildtype and mutant AMLs (Figure 3A). Alternatively, we did identify genes that were significant overexpressed in IDH1 or IDH2-mutated AMLs, and within this list zoomed into the most upregulated PM proteins for which flow-compatible antibodies were available including transmembrane protein 22 (TMEM22), cadherin-2 (CDH2) and the Wnt receptor frizzled class receptor 3 (FZD3) (Figure 3B). Explorative flow analysis of CDH2 and FZD3 in a small subset of IDH1/2 mutant and wild-type patients did not give definitive answers as to whether these proteins would be specifically expressed on IDH1/2 mutated cells, so further studies are required (Figure 3C). Similar approaches for other mutations might further improve the subset of PM proteins by which we can define genetically distinct subclones.

A second challenge is that AML patients sometimes harbor very small subclones with VAFs representing <5% of the total population. These small subclones can preferentially grow out in xenograft mouse models and at relapse of disease making it very relevant to also identify these subclones at initial diagnosis [19, 21]. Identifying these small subclones will require unique and specific PM proteins and novel single-cell techniques like TARGET-seq in order to detect genetic mutations and map relevant cell biological processes in limited available amount of cells [22]. To make bonafide statements it is of the utmost importance to isolate clean AML blast populations, which can be challenging with multiple flow antibodies that have spectral overlap and differ in signal quality. With a single cell “mass cytometry” method named CyTOF you can implement over 30 parameters simultaneously, however, this method does not allow isolation of the cells as viable cell population [23]. A final remark is that PM protein analysis of polyclonal AML patients in some cases resulted in clearly defined distinct subpopulations in Infinicyt-based PCA, but whole exome or targeted Myeloid Trusight sequencing did not reveal VAFs that would fit with the populations we observed by flow cytometry. An intriguing possibility is that in these cases it might be epigenetic changes that underly clonal heterogeneity. Alternatively, it is also quite possible that cell extrinsic differences, for instance positioning of cells within the bone marrow niche, determine the expression of the PM surfaceome.

Altogether, addressing these challenges will further improve the prospective identification and isolation of AML subclones. We should aim to increase the amount of isolated subclones as this might unravel certain communalities and/or differences of subclones with a similar genetic profile.

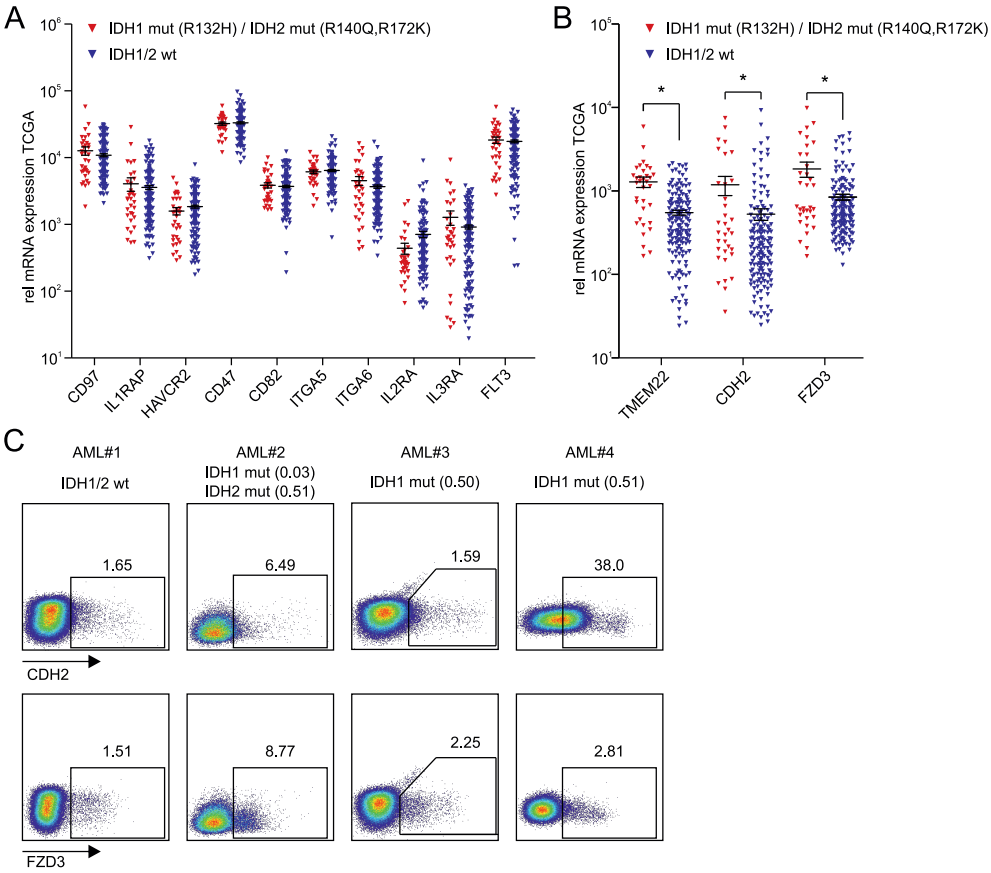

Figure 3. Correlation of PM proteins with IDH1 mutated AML cells

(A) mRNA expression of a subset of leukemia-enriched PM proteins with *IDH1/2* wildtype (wt) and *IDH1/2* mutant (mut) AML patients. (B) mRNA expression of 3 PM proteins with *IDH1/2* wt and *IDH1/2* mut AML patients. (C) Flow cytometry analysis of CDH2 and Frizzled in *IDH1/2* wt and *IDH1/2* mut AML blasts.

Clonal drift in patient-derived xenografts

Patient-derived xenograft (PDX) mouse models enable the study of primary AML patient samples in a more long-term setting that addresses multiple aspects of leukemia development including LSC engraftment in the BM, leukemia outgrowth and dissemination of AML cells to other organs like spleen and liver. Reproducing the subclonal architecture of heterogeneous pre-transplant AML samples in engrafted samples is an important aspect of any PDX model. Previously, we developed a humanized scaffold niche xenograft mouse model that allowed engraftment of 29 out of 39 (74%) primary AMLs [24]. Similar results were obtained by Reinish *et al*., who developed humanized BM ossicles that allowed engraftment of 13 out of 15 (87%) primary AMLs [25]. In both cases, the human microenvironment was superior to murine BM in engrafting good risk AMLs. Both studies partially addressed the subclonal architecture pre-transplant and after engraftment in 3 and 4 AML cases, respectively and

found changes in subclonal composition in at least some of the cases [24, 25]. Changes in subclonal architecture of primary AMLs have been addressed more extensively in NOD scid gamma (NSG) mice. Also, in these models, clonal drift is observed in multiple cases [21, 26, 27]. In contrast, Paczulla *et al*. found relative high numbers of AMLs (18 out of 19) that could engraftment in NSG mice and detected only minimal differences in clonal architecture between pre-transplant and after reconstitution in NSG mice [28]. In the latter study, they performed pre-transplant irradiation, which might augment LSC homing of distinct subclones thereby better preserving clonal architecture. Clearly, the microenvironment plays an important role as well. For example, Antonelli *et al.* demonstrated this for a leukemia patient that harbored a dominant clone with inv(16)/t(9;22) and a minor *IDH1* mutated subclone with a VAF of 0.04 [24]. Injecting sorted $CD34^+$ cell intra-scaffold resulted in engraftment in the scaffold with dissemination to the BM, spleen en liver. Upon exome sequencing of AML cells obtained from the scaffold and murine BM, we noted that the dominant inv(16)/t(9;22) clone engrafted in the scaffold while in the mouse niche only the minor *IDH1* mutated subclone engrafted. Injection of the same polyclonal AML in regular NSG mice without humanized niches only resulted in engraftment in the murine BM in some cases, but the ones that did engraft only contained the minor *IDH1* mutated subclones whereas the dominant inv(16)/t(9;22) subclone did never engraft. These clonal preferences were also observed in NSG mice compared with NSG mice that have knock-in of stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL3) (NSG-SGM3). Here, depending on the presence or absence of human cytokines different subclones engrafted [21]. It has also been proposed that the altered function of the mutated gene(s) in AML can influence engraftability of a subclone. VAFs of founder mutations like *DNA methyltransferase 3A* (*DNMT3A*), *protein tyrosine phosphatase non-receptor type 11* (*PTPN11*) and *RUNX1* were often similar between pre-transplant AMLs and after engraftment in NSG mice whereas mutations in *tumor protein p53* (*TP53*), *NPM1* and *IHD2* where more divergent after engraftment [26].

We were able to identify and prospectively sort genetically distinct subclones as viable populations. This allowed us to study engraftability of genetically distinct subclones of individual patients in the humanized scaffold mouse model [8]. We observed clear preference of subclones in repopulating the humanized scaffold with preferential engraftment of *FLT3-ITD* mutated clones over *NRAS* and *WT1* mutated clones. Although, the numbers are limited, these data together with previously published data indicate that within PDX models clonal drift of polyclonal AMLs is likely to occur. It is therefore important to keep track of the clonal architecture in any *in vitro* and *in vivo* culture of primary AMLs in order to draw the correct conclusions. One could speculate whether this clonal drift is only seen due to limitations of our models or whether similar clonal drift is observed in patients. Quite possibly, the representation of clonal architecture at diagnosis is just a snapshot in time, and most likely

can change significantly as a consequence of time or treatment. Most evidence for the clonal evolution in patients comes from *de novo* – relapse AML patient samples, which revealed that the number of variegating genetic lesions at relapse are increased compared with *de novo* AML and further increases when it takes more time to disease relapse [29, 30]. The majority of these genomic or chromosomal changes at relapse were associated with late proliferative events and not with epigenetic modifying events like mutations in *DNMT3A*, *tet methylcytosine dioxygenase 2* (*TET2*) and *additional sex combs like 1* (*ASXL1*), which were often similar between *de novo* and relapse samples. These data support a model in which the relapse AML is genetically related to the *de novo* AML but that persisting subclones or pre-leukemic hematopoietic stem cells (HSCs) after therapy acquire additional genetic aberrations.

Stability of PM protein expression

Internalization of PM receptors via endocytosis is one of the main mechanisms to switch off or recycle PM receptors [31]. Stable expression at the membrane therefore requires continuous transcription and translation of newly formed receptors. Longitudinal tracking by flow cytometry and specific targeting of genetically distinct subclones requires stable PM protein expression. We analyzed the PM expression at diagnosis and after engraftment in the humanized scaffold (huSC) and murine BM (muBM) (Figure 4A-B) [24]. AML patient #1 was diagnosed with a monoclonal AML harboring an *IDH1* mutation and a FLT3-ITD and this clone could engraft both in the huSC as well as the muBM (Figure 4A). Infinicyt-based PCA on a large subset of leukemia-enriched PM proteins showed one population at the start and one after engraftment in the huSC and muBM (Figure 4A, upper panels). Two representative PM proteins, TIM3 and IL1RAP, were stably expression after engraftment (Figure 4A, lower 2 panels). The second example illustrates the AML diagnosed with a dominant inv(16)/t(8;22) clone and a minor *IDH1* and *FLT3-ITD* mutated subclone as described in the previous paragraph. Here we exclusively observed engraftment of the dominant inv(16)/t(8;22) clone in the huSC and only engraftment of the minor *IDH1/FLT3-ITD* mutated subclone in the muBM. PCA of a large set of PM proteins showed two distinct populations (not genetically different) that were maintained in the huSC whereas engraftment of the minor *IDH1/FLT3-ITD* mutated subclone in the muBM resulted in a single population (Figure 4B, top panel). Two populations were seen based on differential expression of CD82 and ITGA5 *de novo* and after engraftment in the huSC whereas a single cell population was observed in the muBM (Figure 4B, lower 2 panels). Moreover, ITGA5 was similar expressed compared to healthy NBM *de novo* and after huSC engraftment whereas ITGA5 was upregulated in the muBM (Figure 4B, lowest panel). Also PM protein expression analysis of *de novo* – relapse AML patients showed that changes in clonal composition were accompanied with changes in PM protein expression (Figure 4C) [8]. In this case, a patient was diagnosed with a

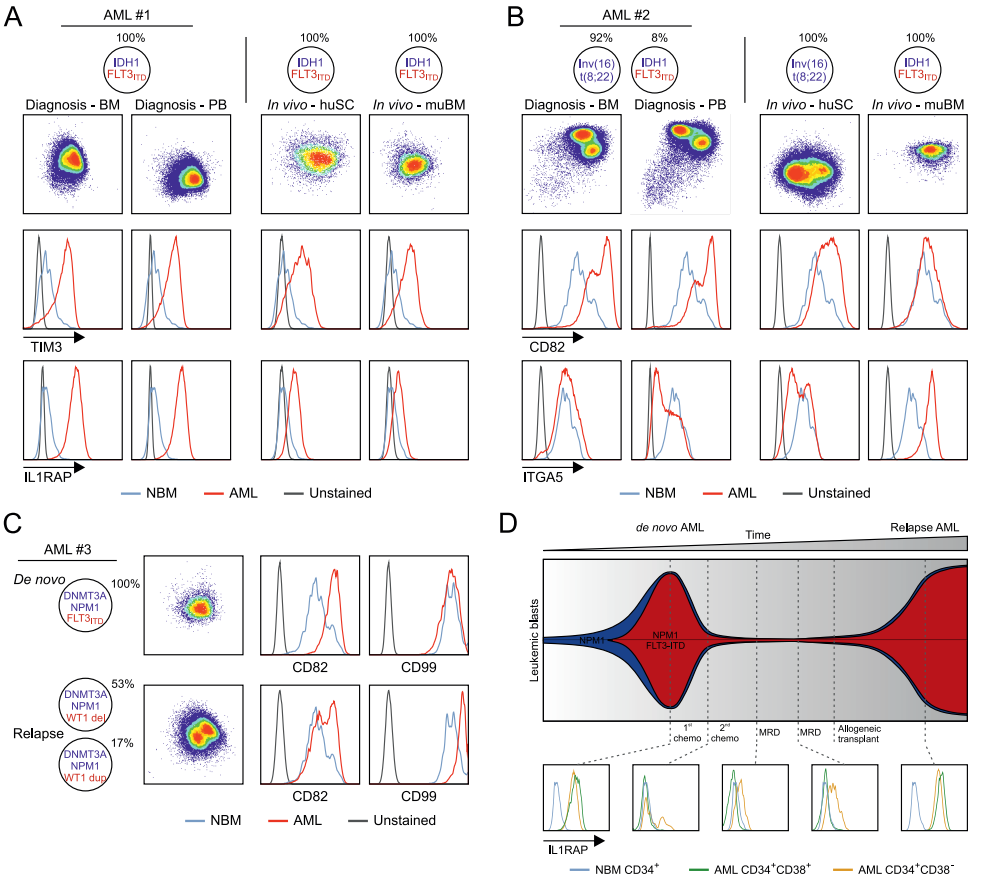

Figure 4. Stably expressed leukemia-enriched PM proteins can define AML (sub)clones in time

(A) AML patient #1 was diagnosed with a monoclonal AML harboring an *IDH1* and *FLT3-ITD* mutation. PM protein analysis and subsequent Infinicyt PCA analysis was performed at diagnosis in BM and peripheral blood (PB) as well as after engraftment in either the huSC or the muBM [24]. (B) AML patient #2 was diagnosed with two subclones, a dominant subclone with an *inv(16)* and *t(8;11)* and a minor subclone with mutations in *IDH1* and *FLT3-ITD*. Only the dominant inv(16)/t(8;11) subclone engrafted in the huSC whereas in the muBM only the minor IDH1/FLT3-ITD mutated subclone engrafted. PM protein analysis and subsequent Infinicyt-based PCA analysis was performed at diagnosis and after *in vivo* engraftment. (C) PM protein analysis in AML patient #3, which had a monoclonal *de novo* AML with mutations in *DNMT3A*, *NPM1* and *FLT3* and a relapse of 2 clones with mutations in *DNMT3A*, *NPM1* and *WT1*^{del}/*WT1*^{dup}. (D) Disease progression of an AML patient that relapsed from the same clone as the one identified in the *de novo* AML. IL1RAP expression was analyzed at different stages in the disease progression.

monoclonal *DNMT3A, NPM1* and *FLT3-ITD* mutated clone and relapsed several years later with a polyclonal AML harboring two subclones with founder mutations in *DNMT3A* and *NPM1* and two different subsequent driver mutations in WT1 including a deletion (WT1 del) and duplication (WT1 dup) (Figure 4C). PCA analysis on leukemia-enriched PM proteins showed a single population in the *de novo* AML whereas two clear distinct populations where observed at relapse that could be separated on the basis of CD82 (Figure 4C). Also,

CD99 expression at relapse was increased compared to the *de novo* AML. Together, these data indicate that expression of PM proteins are rather stable on AML (sub)clones over time, which allows clonal tracking in time by flow cytometry.

This is of importance regarding minimal residual disease (MRD) assessment during complete remission, which allows the identification of small leukemic cell populations. MRD can be addressed molecularly by PCR-based methodologies for specific mutations like *NPM1* and *FLT3-ITD* and with multiparametric flow cytometry of aberrant PM protein expression [32, 33]. Challenges in MRD detection include PCR sensitivity and the availability of leukemia-specific PM proteins. IL1RAP is specifically expressed in leukemic blasts and stem cells and would be a good candidate to detect MRD. Therefore, we longitudinal followed an AML patient that was diagnosed with an IL1RAP$^+$ clone (Figure 4D). Complete remission was established after the 1st treatment, however, a very small population of IL1RAP$^+$ within the CD34$^+$CD38$^-$ compartment remained. In time, this IL1RAP$^+$ population increased in size and ultimately this patient relapsed from the similar clone as seen at first diagnosis. Current ongoing experiments are aimed at isolating such MRD$^+$ cells using flow-sorting followed by (targeted) sequencing to prove that these cells are indeed leukemic and not healthy wild-type cells that express aberrant PM markers, for instance as a consequence of the regeneration process upon transplantation.

Immunotherapy treatment of AML patients

Immunotherapy has become part of possible treatment strategies for many different cancers, primarily based on successes of antibody-medicated checkpoint blockade via cytotoxic T-lymphocyte–associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD1), discoveries for which James Allisson and Tasuku Honjo received the Nobel Prize in Physiology and Medicine in 2018 [34, 35]. Meanwhile, alternative immunotherapy approaches were developed including antibody-drug conjugates (ADCs), bi-/trispecific antibodies and chimeric antigen receptor (CAR) T cells [36-38]. ADCs comprise a monoclonal antibody coupled to a cytotoxic payload, which is internalized upon binding to the cognate antigen and release of the cytotoxin, causing cell death [36]. Bi-/trispecific antibodies are capable recognizing multiple distinct antigens, at least one epitope often recognizes cytotoxic T cells whereas the other epitope(s) recognize tumor antigens [37]. Finally, CAR T cell therapy requires T cells that are engineered to express tumor antigen recognition receptors, in addition, these T cells have been further modified with co-stimulatory signaling domains and T cell activating components [38]. All these different strategies have one common denominator, they heavily depend on the specificity and accessibility of the chosen antibody target(s).

Also, in AML treatment, immunotherapy is a promising new therapy and a lot of early phase clinical trials are currently conducted, although with varying results [39]. A major complication in treatment of AML patients with immunotherapy are off-target effects, as

many proposed tumor antigens are also expressed on healthy stem and progenitor cells [39]. A lot of effort has been made to identify targetable antigens specifically expressed on AML cells, which resulted in various possibly candidates including CD123 (also known as IL3 receptor alpha IL3RA), TIM3, CD44, CD47, CD96, CD99, C-type lectin-like (CLL1) (also known as C-type lectin domain family 12 member A (CLEC12A)) and IL1RAP [1, 40-46]. Unfortunately, none of these markers have made it into clinical practice as target for immunotherapy so far. In part this is due to off-target toxicity but possibly also as of the considerable amount of heterogeneity seen within individual AML patients, whereby not all subclonal tumor cells would express the antigen. The identification of known but also new leukemia-enriched PM proteins by full quantitative proteomics in our study might contribute to improved immunotherapy [8]. Perna *et al.* carefully addressed the expression of potential candidates in a wide variety of non-hematopoietic tissue in order to predict off-target toxicity [7]. They propose 30 candidate PM proteins of which CD44, CD82, CD96, CD123 and CLEC12A overlap with our list of 50 leukemia-enriched PM proteins. Still many proposed candidates are enriched in AML but not per se specific for AML. The identification of co-expression of multiple leukemia-enriched antigens on the same AML cell might improve immunotherapy as strategies that make use of multiple antigen recognition sites like bi/tri-specific antibodies might have reduced off-target toxicity. In our case, genetically distinct subclones could often be detected and separated on the basis of more than one maker, which would allow targeting of these subclones with multiple enriched tumor antigens. A combination of patient-specific and in some cases subclone-specific drugs might be needed to prevent relapse of disease.

AML cells within the BM microenvironment

The BM microenvironment is critically important for healthy hematopoiesis as well as leukemogenesis [47, 48]. The interaction with the niche cells can either be direct, via adhesion molecules, or indirect via secreted growth factors, chemokines and extracellular matrix molecules. It has been demonstrated that *in vitro* culturing of immature hematopoietic stem/progenitor cells (HSPCs) on a confluent layer of murine marrow stromal cell line (MS5) or human mesenchymal stem cells (MSCs) of early passages improved long-term proliferation compared to liquid culture conditions [49, 50]. Primary AML cells grown on a stromal layer of MSCs showed heterogeneous but on average increased secretion of cytokines including IL1β, tumor necrose factor-α (TNFα), chemokine (C-X-C motif) ligand 1 and 5 (CXCL1/5), matrix metalloproteinase-1 (MMP1), granulocyte colony-stimulating factor (G-CSF), and GM-CSF [51]. This cytokine milieu supported long-term growth and inhibited apoptosis of primary AMLs. Also, the use of MSCs in humanized xenograft mouse models resulted in improved AML engraftment, as described before [24, 25]. Remaining challenges of *in vivo* xenografts include the absence of species-specific growth factors like G-CSF, thrombopoietin

(TPO) and IL3 that are important for leukemogenesis and LSC maintenance. In addition, it has been shown that lineage fate of mixed lineage leukemia (MLL)-rearranged leukemia is affected by the cell of origin, its fusion partner but also the BM cytokine milieu [52-54].

Characterization of the MSC secretome by transcriptome analysis as well as cytokine arrays revealed that especially TPO and IL3 are not secreted by MSCs [55, 56]. The lack of these cytokines, IL3 in particular, might explain the bias along the lymphoid lineage in a cord blood (CB) $CD34^{+}$ MLL-AF9 humanized BM scaffold xenograft model [57]. Therefore, we genetically engineered human MSCs to overexpress IL3 and TPO as alternative for human cytokine expressing mice like the macrophage colony-stimulating factor (M-CSF), IL3, GM-SCF, signal regulatory protein α (SIRPα), TPO, recombination activating gene 2 $(RAG2)^{-/-}$ interleukin-2 Receptor Subunit Gamma $(IL2Rg)^{-/-}$ (MISTRG) and NSG-SGM3 mice [55]. We indeed observed for the first time a full myeloid MLL-AF9 leukemia in PB, BM, spleen and liver, however, the majority of mice still showed lymphoid output and the tumor formation in the humanized cytokine scaffold was reduced compared to the previously established humanized scaffolds. Genetic overexpression of IL3 and TPO in primary MSCs does not allow fine tuning of the local cytokine levels to the desired physiologically relevant concentrations. The reduced engraftment in the scaffold might be caused by high levels of IL3 within the scaffold driving differentiation of LSCs and thereby loss of self-renewal and scaffold engraftment. LSCs that migrate to the mouse compartments directly after injection might escape these high local levels of human IL3 within the scaffold and thereby retain their self-renewal capabilities. Subsequently, these LSCs can reconstitute the murine compartments where their progeny showed a slight increase in differentiation along the myeloid lineage in some cases, which might be as of circulating human IL3 in the mouse blood that has been produced within the scaffold. Alternatively, it has been shown that the developmental stage of the human niche also influences lineage fate of MLL-rearranged leukemias [58]. Comparison of neonatal and adult BM stromal cells showed that chemokine (C-C motif) ligand 5 (CLL5) is specifically secreted by adult stromal cells forcing a bias along the myeloid lineage in adult stromal conditions. [58]. In our studies we made use of one adult MSC donor across all different experiments, however, we still maintained a bias along the lymphoid lineage. Clearly, these data show that lineage fate of MLL-rearranged leukemia is not only dependent on external cues like IL3 but other factors including cell source, age-dependent (adherent and secreted) BM niche factors and fusion partners influence lineage outcome. Likely we need multiple different models to fully recapitulate all different types pediatric and adult MLL-rearranged leukemias.

The role of IL1RAP in the inflammatory BM microenvironment

Inflammation is a tightly regulated immune response to an irritant. Different mechanisms of hematopoietic activation upon stressors have been proposed including activation by pro-

inflammatory cytokines like type I interferons (IFNs) and activation of toll-like receptors (TLRs) and Nod-like receptors via pathogen-associated molecular patterns (PAMPs) [59]. This natural response provides us with an complex defense mechanism against pathogens and other stressors, however, prolonged and excessive exposure to these inflammatory milieu can actually promote BM failure and initiation of hematological malignancies including myelodysplastic syndrome (MDS), myeloproliferative neoplasm (MPN) and AML [48, 60-62]. In this thesis we studied the role of IL1RAP, an important IL1 signaling receptor in regulating inflammation, which has been shown to be specifically expressed in blasts and stem cells of CML, MDS and AML patients compared to healthy HSPCs [46, 63, 64]. IL1-IL1RAP signaling resulted in downstream activation of MAPK p38 pathway and subsequently NFκB in primary AML cells. NFκB is a transcription factor, which is frequently observed constitutively active in AML and can upregulate different anti-apoptotic proteins but also is a key regulator of the inflammatory response via upregulation of multiple chemokines and cytokines [65-67]. In contrast to previous findings, we did not observe an increased survival under stress conditions [68]. Neither did knockdown of IL1RAP result in increased apoptosis and thereby reduced viability as was proposed previously [46, 68]. We observed similar results in a shRNA library screen of KBM7 cells *in vivo* where knockdown of IL1RAP with 5 different shRNAs did not result in loss of engraftability and survival. The data in this thesis would suggest that IL1RAP does not directly have a cell intrinsic role in leukemia cells.

We found that the IL1-IL1RAP induced secretome of AML cells affects healthy hematopoiesis and not AML cells themselves. Interestingly, the effect was only seen when AML cells were grown in the context of MSCs suggesting that the cross-talk of AML cells with their microenvironment is essential for this effect. Also, MSCs express IL1RAP and upregulate several chemokines upon stimulation with IL1β, however, this had limited effect on healthy hematopoiesis compared to the secretome of AML-MSC co-cultures. The importance of cross-talk between AML cells and MSCs has been addressed previously. Genome-wide gene expression analysis of MSC in co-culture with AML cells showed increased expression of genes involved in NFκB, TLR and chemokine signaling including interleukin-6 (IL6), IL1B, interleukin-1 receptor-associated kinase-like 2 (IRAK2), interleukin-8 (IL8) and CXCL1 [69]. In addition, Carter and colleagues showed that IL1β can induce cyclooxygenase-2 (COX2)-mediated upregulation and secretion of prostaglandin E2 (PGE2) in MSCs [70]. In turn, PGE2 secretion results in β-Catenin-mediated upregulation of caspase recruitment domain, an anti-apoptotic protein, in AML cells [70, 71]. The secretome of AML-MSC co-cultures resulted in reduced healthy hematopoietic cell numbers, however, at early timepoints the immature $CD34^+$ HSPCs remained unharmed. These data would suggest that HSCs are less affected by this inflammatory secretome compared to more differentiated cells. Miraki-Moud and colleagues showed that healthy mouse HSCs in the BM of AML patients had a differentiation block but remained unharmed themselves [72]. Isolation and analysis of these HSCs showed

that they kept their stem cell features and were able to differentiate along the different lineages suggesting that this stop in differentiation is reversible. Interestingly, it has also been shown that PGE2 can result in HSPC survival and expansion in a similar manner as observed for AML cells [73]. In our assay, we could not detect persisting $CD34^+$ cells for long periods of time, but more functional assays including colony forming cell assays at later timepoints would be important to determine whether progenitor activity would be maintained.

The IL1-IL1RAP signaling axis in AML might also play an role in clonal hematopoiesis of the elderly and AML initiation as upon ageing the BM microenvironment suffers from chronic inflammation with higher basal levels of pro-inflammatory cytokines like IL6, TNFα and IL1β [62]. This chronic inflammatory BM environment results in accumulated DNA damage in activated HSCs thereby result in pre-leukemic HSC clones as an early step in leukemia development [74]. The presence of clonal hematopoiesis increases the risk to develop a hematological malignancy but it is not fully clear if and how founder mutations in *DNMT3A*, *TET2* and *ASXL1* would contribute to this [75]. It has been shown that *Tet2* deficient mice have increased stem cell self-renewal and have an increased risk to develop pre-leukemic myeloproliferation *in vivo* [76]. In addition, inflammatory signals including IL6 appear to be crucial for this myeloproliferative phenotype observed in *Tet2* deficient mice [77]. Similar findings have been made in *Dnmt3a* deficient mast cells, which secreted higher levels of IL6, TNFα and interleukin-13 (IL13) [78]. In addition, *Dnmt3a* loss together with a *Jak2* V617F mutations activated inflammatory signaling in HSPCs [79]. Clearly, inflammation can play a role in leukemia initiation but it remains unclear whether also IL1-IL1RAP signaling plays a role in this. In summary, both the intracellular molecular state determined by mutations associated with clonal hematopoiesis as well as the inflammatory state of the BM niche are likely affecting the IL1RAP expression on the outer membrane. It will be important to take both these features into account if we use IL1RAP to target AML cells and to study MRD as it might be difficult to distinguish regenerating hematopoiesis from residual leukemia in a by chemotherapy disturbed BM niche.

Concluding remarks

Clonal heterogeneity of AMLs in one of the major challenges in current AML treatment. Within this thesis, we identified leukemia-enriched PM proteins that allow the identification and prospective isolation of genetically distinct AML subclones. We showed that subclones rely on different biological processes and require different treatment strategies. In addition, the isolation of subclones as viable cells provide unique opportunities for future studies that study clonal heterogeneity in AML. This will improve our knowledge of subclonal evolution from CHIP up to relapsed AML. If we can find subclone-specific vulnerabilities we can improve therapy strategies thereby prevent relapse of disease.

In addition to this, we further improved our previously established xenograft mouse models that allows studying of leukemia clones in a physiological more relevant way. We also provide new evidence for the function of the leukemia-specific surface marker IL1RAP. IL1-IL1RAP signaling in AML cells induced an inflammatory secretome, which was detrimental for healthy hematopoietic cells leaving leukemogenesis intact. Targeting this signaling might improve hematopoietic recovery over leukemogenesis.

It will be of importance to increase the number of patients in which we can segregate subclones as viable cell populations as this will allow us to make statements regarding relapse frequency, treatment sensitivity and competitiveness of individual subclones. Clonal tracking, the isolation and functional characterization of subclones at different time points will improve our knowledge of disease progression from CHIP to MDS/AML as well as progression into a relapsed leukemia. Improving the humanized scaffold niche xenograft mouse model by introducing endogenous human growth factors will provide us tools to study clonal evolution *in vivo*. The ultimate goal is to provide patients with a curative personalized subclone-specific treatment that prevents relapse of disease.

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